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TRACHEOBRONCHIAL MUCINS IN HEALTH, DISEASE, AND TOXIC EXPOSURES

FINAL REPORT

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The major portion of this project, identification of mucin core peptide, raising the antibody against mucin peptide, mucin gene expression in rat tracheal epithelial culture system, has been successfully accomplished. The main discovery in this project is the role of retinoic acid (a component of vitamin A) in maintaining healthy tracheal epithelial cells exposed to different toxic and carcinogenic substances, and use of steroids in suppression of mucin gene expression. Further studies are underway to discern the mechanism involving the role of vitamine A in mucin gene expression in the tracheal epithelial cell system. The environmental research facility and exposure chamber have been completed. Experiments relating to exposure will begin shortly. The last portion of the project could not be accomplished due tc the delay in the contracting procedures relating to construction of the environmental facility and exposure chamber.

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FOREWORD

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INTRODUCTION

PROBLEM TO BE STUDIED:

The respiratory tract of animals, including humans, is constantly exposed to the external environment and is the main port of entry for potentially hazardous environmental agents. Many of the agents which are encountered are toxic chemicals, irritant gases (e.g., CO, O_3, SO_2, NO_2, NH_3, etc.), airborne particles, and biological agents. When inhaled, these toxic substances first come in contact with the epithelial lining of the airway, then macrophages, alveolar cells, and finally affect the capillary system of the alveoli. These inhaled substances (both particulates and biological agents) deposit on the mucociliary tracheal epithelium and alveolar cells. These agents can then be removed by the mucociliary escalator system or by the epithelial cells.

Different studies, including epidemiologic data, with laboratory animals indicate a definite link between toxic exposures and acute as well as chronic respiratory diseases, including cancer. The tracheobronchial tree responds to various noxious agents and in different allergy-induced diseases like bronchitis and asthma, certain gene-linked diseases like cystic fibrosis, virus-infected diseases like AIDS, and chemical carcinogen-induced diseases like cancer, by increasing the volume and changing the nature of its protective barrier - mucus. Although the physiological control of excessive production of mucus is not fully understood, cholinergic, alphaadrenergic, and beta-adrenergic stimuli have been suggested to contribute significantly to the problem. In addition, respiratory mucus secretion may be due to hypersensitivity or inflammatory responses to a number of mediators (such as, histamine and cyclooxygenase), including components released from phagocytic cells (e.g., macrophages, monocytes or neutrophils). At present, once the pathological changes are established, there is little we can offer to the patients. It is one aim of this proposal to provide a better understanding of the control mechanism of tracheobronchial mucus secretion on the cellular as well as genetic level both under normal, healthy conditions and during episodes of disease or toxic exposure. Such an understanding will lead to new approaches in the prevention, control and reversal of mucus secretion. Clearly, this is a field where medical scientists, by in-depth probing through detailed molecular analysis, can link with clinicians, helping to control secretion of mucus in the prevention or cure of disease and environmental insults.

For several years, efforts have been made to find effective pharmacological agents for the chemoprevention of different kinds of cancer in humans, some of which are caused by toxic or carcinogen exposure. Among these agents, retinoids (vitamin A and its components) have been known to play an important role in preventing metaplasia and regulating cell growth and differentiation of various epithelial cells, including tracheal epithelial cells. When vitamin A is not present in the diet of experimental animals, mucus-producing cells undergo squamous metaplastic transformation. This transformation has been found especially in tracheobronchial epithelia of vitamin A-deficient animals. A decrease in secretion of mucus has been

observed simultaneously with the development of squamous metaplasia. Recently, we have found that rat or rabbit tracheal epithelial cells, when cultured in serum-free and hormone-supplemented medium, require retinoic acid for optimum proliferation and without this reagent, the cells did not grow nor secret any mucin -- major component of mucus. In rabbit tracheal cell lines, mucin gene expression was only observed when the cells grew in the presence of retinoic acid. Also, we observed that rat tracheal cells did not express mucin gene initially, but retinoic acid, when added to the culture medium, induced expression of this gene, suggesting that vitamin A and its components are partially responsible for the healthy growth of the epithelial cells. In chemically induced carcinogenesis of bladder,mammary gland and tracheal epithelium of different animals, retinoic acid has been found to have a protective effect. However, the mechanism involved in the protective action of retinoids has not been thoroughly investigated.

Another pharmacologic approach in controlling mucus hypersecretion is to use drugs, like tetracycline and antihistamines, before using corticosteroids and atropine. But the remission is temporary and the underlying mechanism involved in the action of these drugs is not fully understood. One of the main objectives of this project is to study the effects of the drugs on the synthesis of mucin at the gene level in a tracheal organ or cell culture system.

For the last several years, our studies at the Department of Clinical Investigation, William Beaumont Army Medical Center, El Paso, Texas, have been concerned with the characterization and synthesis of respiratory mucins. We have achieved the first goal by partially characterizing the core protein of human tracheal mucin and raising an antibody in rabbit against this protein. We have been successful in isolating tracheal epithelial cells from different animals (such as rabbits and rats) and maintaining them in culture for several weeks. These cells have been found to secrete mucin in the culture medium, thus offering a system to study the control of the secretion of mucin at the cellular and particularly at the genetic level. We have already characterized the mucin gene from these cells, and experiments are under way to examine the expression of this gene in the presence of different chemical agents and therapeutic drugs, and in animals exposed to toxic exposures.

OBJECTIVES:

In view of the above observations, our specific aims in this proposal are:

a. Study the levels and control of transcription and mucin mRNA accumulation in tracheal epithelial cells and organ culture systems of animals exposed to various noxious agents (such as SO_2, NO_2,and NH_3),and assess the protective action of different therapeutic drugs, including steroids and retinoids (vitamin A), against these exposures;

b. Study the control of mucin gene expression in chemically induced carcinogenesis of the tracheal epithelium in rats and mice, and the protective action of retinoids (vitamin A and its analogs) and other therapeutic drugs against the effects of these compounds. Some of the toxic componds which will be used are: N-methylN-nitrosourea (MNU), dimethylbenz(a)anthracene (DMBA), N-methyl-N'-nitro-Nnitrosoguanidine (MNNG), and N-butyl-N-(4-

hydroxybutyl) nitrosamine (BBN), the latter compound being a derivative of nitrosamine. Nitrosamine is used as a fuel in military hardware systems. Once the effects of these compounds on the respiratory tract system, especially the production of mucin, are understood, a regimented treatment, including retinoid therapy, can be proposed for subsequent study; and

c. Generate an immortal mucin-producing cell line by transfecting the primary tracheal epithelial cells with different non-pathogenic viruses and agents known to produce transformed cell lines. These cell lines will provide us with systems to study the long-term effects of toxic agents and drugs on the increase in production or inhibition of mucins, both on cellular and genetic levels.

BODY

Characterization of human mucins and preparation of antibody:

Mucous from humans suffering from asthma and chronic bronchitis was collected in a tube containing proteolytic inhibitors. The mucous was solubilized in water containing 1 mM of sodium azide at 4°C for 24-72 hours. After removing the insoluble material by centrifugation, the water-soluble material was chromatographed on differentially sized exclusion column to isolate mucins. The mucins appeared at the void volume of the column. The mucins were separated into neutral and acidic fractions by ion exchange chromatography. Amino acid and carbohydrate analysis of the purified material indicated a typical mucin composition in that there were high levels of threonine, serine, proline, all the carbohydrates including galactosamine, but excluding mannose. The mucins were deglycosylated by established procedures and different peptide fragments were fractionated by high pressure liquid chromatography. The major peptide had a molecular weight of about 90 Kb with a composition characteristic of typical mucin. This peptide was used to raise antibodies in rabbits. The antibody was found to react with mucins isolated from different sources, such as human intestine, colon, milk and rat trachea. This antibody will be utilized to monitor mucin protein synthesis in tracheal epithelial cells in culture.

Culture of tracheal epithelial cells:

Before the initiation of this project, rabbit tracheal epithelial cells were isolated and cultured on collagen-coated dishes in a serum-free and hormone-supplemented medium containing retinoic acid. The cells required retinoic acid to grow properly. Without retinoic acid, the cells did not propagate to form a monolayer, nor did they produce mucins. The normal tracheal epithelial cells grown in the presence of retinoic acid produced mucins, the compositions of which were comparable to those of human mucins. The secretion of mucins was monitored by [^{3}H]glucosamine incorporation into the carbohydrate moieties. Addition of different chemical and toxic agents, (aryl- N-acyl-galactosaminides, 5-azacytidine and N-nitrso-N-ethyl urea) and pharmacologic agents (histamine, methacholine, atropine, pilocarpine and isoproterenol) into

culture medium resulted in either augmentation or inhibition of mucin production. The ability of this tracheal culture system to synthesize mucins was further demonstrated by its ability to produce the mucin gene. Total RNA isolated from these cells at different periods of growth hybridized with an antisense 30 base oligonuleotide corresponding to a rat intestine mucin protein sequence.

After the preliminary studies as stated above, we attempted to culture rat tracheal epithelial cells in the same serum-free and hormone-supplemented medium containing retinoic acid. Though the cells propagated normally, they did not form a monolayer, which is characteristic of typical epithelial cells. We were unsuccessful in monitoring the secretion and sythesis of mucins in these cell lines. Thus, instead of working with isolated epithelial cells, we diverted our attention to a rat tracheal organ culture system. This system allowed us to study the effects of retinoic acid and different chemical (toxic and non-toxic) and pharmacologic agents on the expression of mucin message. Ultrastructural examination of rat tracheal organ cultures, maintained in a serum-free and hormone-supplemented medium containing retinoic acid, exhibited a well-preserved respiratory epithelium for at least 196 hours. Hybridization analysis using a 30 base oligonucleotide derived from a rat intestinal peptide tandem repeat sequence demonstrated that the cultures were expressing mucin gene. The organ cultures were also found to secrete mucins into the medium. Northern hybridization of rat tracheal total RNA with the same probe demonstrated a mucin mRNA with a size of 7.5 Kb. In the absence of retinoic acid, the cells stopped producing mucin message and addition of retinoic acid to the deficient medium resulted in appearance of hybrization signal. Short-term incubation with pharmacologic agents (methacholine, phenylephrine and histamine) showed stimulating effect on mucin gene expression, whereas, atropine, pilocarpine and isoproterenol had little effect. The increase in mucin gene expression was blocked by atropine. 8-bromo cyclic AMP and 5-azacytidine were found to enhance mucin gene expression. The steroid prednisolone inhibited the expression of mucin message. The expression of mucin gene was not enhanced when the organ culture was treated with the chemical carcinogens N-methyl-N-nitrosourea and phorbol 12-myristate 13-acetate for 96 hours. However, incubation of tracheal culture with N-methyl-N-nitrosourea for 24 hours resulted in an increase of hybridization signal; withdrawal of the drug after initial exposure resulted in a decrease of intensity, indicating that the agent, once within the cells, had a long-term effect on mucin message and short-term incubation with retinoic acid had no visible effect on this system. Studies are being performed to assess the long-term effect of incubation with retinoic acid on the drug-induced inhibition of mucin message. Studies are also underway to elucidate the control of regulation of mucin message on the transciptional level in tracheal epithelial cells grown with or without retinoic acid. Another study which will be undertaken shortly is to examine the effect of retinoic acid on rats exposed to different toxic gases, such as sulfur dioxide and nitrogen dioxide. The installation of an exposure chamber has just been completed.

Thus, our discovery that retinoic acid, a component of vitamin A, is absolutely necessary to propagate and maintain healthy tracheal epithelial cells and has a protective effect on normal function of these cells, such as

secretion of small amount of mucous to maintain the mucocilliator- escalator system, exposed to different toxic substances, may have far-reaching significance with respect to different respiratory diseases, such as chronic bronchitis, asthma and cystic fibrosis, amongst the general population as well as military personnel.

CONCLUSIONS

The major portion of this project, identification of mucin core peptide, raising the antibody against mucin peptide, mucin gene expression in rat tracheal epithelial culture system, has been successfully accomplished. The main discovery in this project is the role of retinoic acid (a component of vitamin A) in maintaining healthy tracheal epithelial cells exposed to different toxic and carcinogenic substances, and use of steriods in suppressing mucin gene expression. Further studies are underway to discern the mechanism involving the role of vitamin A in mucin gene expression in the tracheal epithelial cell system. The environmental research facility and exposure chamber has been completed. Experiments relating to exposure will begin shortly. The last portion of the project could not be accomplished due to delay in the contracting procedures relating to construction of the environmental facility and exposure chamber.